REMARKS

Status of the Claims

Claims 25, 27-29, and 31-38 are currently pending. Claims 25, 29, 31 and 32 are amended. Claims 26 and 30 have been canceled. New claims 36-38 are added. See *infra* for support for amendments and new claims.

English Translation

The Patent Office acknowledges the priority claim to Korean Patent Application No. 2000-13161, filed March 15, 2000, although an English translation must be submitted to perfect the priority claim.

Accordingly, submitted herewith is an English translation of Korean Patent Application No. 2000-13161 to perfect the priority claim.

Additional Restriction Requirement

Applicants previously elected claims 25-35 for prosecution in this application. Applicants also traversed the further restriction to *single* HPV nucleic acid probe and a *single* pair of primers. The Examiner contends that since the sequences of the various HPV strains differ structurally, their presence on an array would constitute separate and independent inventions.

Applicants did not, and still do not, find this additional requirement well-founded or even within the scope of a permissible restriction requirement, and it is therefore **traversed**. The present invention is **not** attempting to claim the individual, disparate HPV nucleic acids. To the contrary, the purpose of the instant invention is to identify *which* of the disparate HPV strains is present in a biological sample. In order to do this, it is necessary to have an array of HPV strains to which a sample DNA can be evaluated for hybridization. Therefore, restricting the array to a single probe would essentially **defeat the purpose of the invention** since it would necessitate use of about 20 separate arrays per single sample to achieve the object of the invention, i.e., diagnosis of an HPV

strain. This would be prohibitively expensive and time consuming and not at all practical from a diagnostic standpoint. It would also require significantly more sample from patients. It appears there may be a misunderstanding on the Examiner's part regarding the nature of the invention.

It is submitted that the Examiner does not need to search individual sequences in order to examine the present claims. The Examiner has not yet cite any prior art that is directed to a **microchip** comprising only HPV nucleic acids, so it is presumed that no such array was known until the present invention. Accordingly, no searching can required for specific HPV sequences which would be present on an array, since the array is non-existent. It is reiterated that the claims are not drawn to individual HPV sequences, or primers, which Applicants acknowledge were previously known, instead, the claim is to a diagnostic method using the sequences.

For support, Applicants' respectfully direct the Examiner's attention to issued patents with claims to microarrays, or use thereof, which disclose multiple sequences, some non-specified, on arrays, e.g., U.S. 6,511,849 and 6,709,855.

Instead, claim 25 is currently amended to recite that the HPV nucleic acid sequences on the chip comprise HPV SEQ ID NOs: 1-19. According to U.S. patent law, the claims must precisely recite the individual components of a kit, including the nucleic acids which comprise the array. It is respectfully submitted that the claims, as amended herein, comply with that requirement.

Accordingly, withdrawal of this requirement is respectfully requested.

However, in order to avoid abandonment of this application since the statutory deadline is today, Applicants ***provisionally*** elect the probe for HPV16 (SEQ ID NO: 1) and primer pair consisting of SEQ ID NOs: 22 and 23. However, Applicants' agent respectfully requests a telephone interview with the Examiner to discuss the propriety of restriction requirement further.

Rejections Under 35 U.S.C. §112: Indefiniteness

Claims 25-35 have been rejected as indefinite for reciting the phrase "to give biotin-containing amplified DNA". The Examiner notes that there is no antecedent basis for "biotin". In addition, the Examiner alleges that claim 25 is indefinite because it does not provide a relationship between the detection of the bound DNA and the method for diagnosis. Further, the Examiner contends that claim 25 is indefinite because it is not clear if there is a second label involved in the method in addition to the biotin-containing primers.

To address the Examiner's rejections, claim 25 has been amended to more accurately claim the present invention. Specifically, "to give biotin-containing DNA" has been replaced with "to obtain biotin-containing" DNA; the lack of antecedent basis for "biotin" has been obviated by the addition of the term "biotin-containing" to refer to the primers in step (ii); a phrase has been added to the end of the claim to provide a relationship between detection of the bound DNA and diagnosis; and reference to a second label that binds to biotin has been included as new step (c) to clarify that there is indeed a second label that binds to the first label.

In addition, claims 26 and 30 have been canceled since the subject matter of these claims is now in claim 25.

Support for these amendments can be found in the specification matter is incorporated in to claim 25, and claim 31 has been amended to depend from claim 25 instead at page 8, steps 1 and 3, and example 2-2 and 2-3 on page 12. Specifically, page 8 indicates that streptavadin-R-phycoerythrin is the preferred means for the second label-the application is not limited to the second label being this, but can be any label that binds biotin.

In addition, new claim 36 has been added which recites similar subject matter as claim 25, but does not limit either the first label or the second label. New claims 37-38 further specify the nature of the labels. This amendment is support by the specification in the "Summary of the Invention" on page 4 and the "Detailed Description" on page 6 which refer to "means for labeling" DNA and probes. It is respectfully submitted that means for double-labeling nucleic acids was

well-known to a skilled artisan in 1998, as indicated by attached Exhibits A and B (abstract by Ramsay and abstract by Solanas et al.) which refers to numerous labels for DNA including fluorescent labels and digoxin which are contemplated for use in the presently claimed method..

No new matter is added by these amendments.

In addition, the Examiner rejects claim 28 for the term "position markers". The Examiner asserts that since the term position markers is not defined in the specification, the meaning is unclear.

To address this rejection, Applicants' respectfully direct the Examiner's attention to the specification at page 13, line 28, which indicates that the position markers are for locating specific probes on the DNA chip. "Position markers" is an art-related term that is understood by those skilled in the art of microarray technology as indicated by the article at Exhibit C, by Antonio et al., published in 1995 (see Figure 1 on pages 35-36 Table 1 on page 37 which refer to position markers or DNA markers).

Rejections Under 35 U.S.C. §103: Obviousness

Claims 25, 28 and 30 stand rejected as obvious over **Gravitt** et al., J. Clin. Microbiol. 1998; 36; 3020-27 ("Gravitt"), in view of an excerpt from the **Stratagene** catalog from 1988 ("Stratagene").

The Examiner contends that Gravitt teaches a method of genotyping/diagnosing HPV by labeling amplified DNA attached to a solid surface, and that Stratagene teaches that reagent kits for hybridization were conventional in the art as of the filing date of this application. The Examiner therefore asserts that it would have been obvious to one of ordinary skill in the art to provide an HPV hybridization kit comprising a DNA chip (which is a solid surface).

A *prima facie* case of obviousness must meet three criteria: First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally

available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Third, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure. *In re Vaeck,* 947 F.2d 488, 20 U.S.P.Q2d 1438 (Fed. Cir. 1991). The determination of obviousness, as enunciated in *Graham v. John Deere Co.*, 383 U.S.1 (1966), rests on four factual inquiries: (i) determining the scope and contents of the prior art; (ii) ascertaining the differences between the prior art and claims in issue; (iii) resolving the level of ordinary skill in the pertinent art; and (iv) evaluating evidence of secondary considerations. Also, a finding of obviousness requires that there be a concrete suggestion or motivation to modify what is taught in a reference, or to combine its teachings with other references, and the combined or modified prior art must actually teach all of the claimed limitations. Both the motivation and the reasonable expectation of success must be found in the prior art and not in Applicants' disclosure.

The combination of Gravitt and Stratagene **do not** meet these criteria for the following reasons. First, Gravitt does not teach or suggest a solid- surface DNA chip, much less a diagnostic kit. Gravitt employs diffusible *nitrocellulose* filters or *nylon* strips for dot blotting or line blotting hybridization, and asserts an advantage of this method over the prior art. Please note that Gravitt was published in 1998, when chip-based microarrays were already known in the art. While Gravitt's line blotting method uses biotinylated DNA primers containing dUTP to generate labeled DNA, followed by colorimetric detection with streptavidin-peroxidase conjugate, Gravitt neither discloses non-diffusible chips nor a suggests a diagnostic kit. The Stratagene reference does not supply the missing teaching, *i.e.*, there is **no mention** of a DNA chip or hybridization/diagnostic kits. The Stratagene kits referenced are directed to nucleic acid sequencing, RNA transcription, *in vitro* translation, and immunoscreening, none of which are useful for diagnostic purposes. Accordingly, there could have been no motivation to combine the two references, and thus, no finding of obviousness. Moreover, had Gravitt intended his technology to be applied to a microarray chip, he would have done so since the chips were well known and used.

Claims 26 and 29 stand rejected as obvious over Gravitt in view of Stratagene, and further in view of PCT international application **WO 95/22626** ("the PCT"). The Examiner's rejections over Gravitt and Stratagene are the same as above, and he further contends that the PCT teaches an HPV 16 sequence probe identical to instant SEQ ID NO: 31, as well a primers having sequences identical to instant SEQ ID NOs: 24 and 25. In the Examiner's opinion, it would have been obvious to use the probe and primers of the PCT to make a DNA chip-containing kit for HPV diagnosis, as allegedly taught by Gravitt and Stratagene.

As discussed above, absent a teaching of a solid-surface DNA chip, or a suggestion to substitute the nitrocellulose/nylon filters of Gravitt with a glass chip as components of a diagnostic kit, the HPV probe/primers of the PCT do not overcome the deficiency in the Examiner's obviousness argument.

Claim 27 was rejected as obvious over Gravitt in view of Stratagene, further in view of **Bevan** et al., Biochem. J. 1990; 267: 119-23 ("Bevan"). The sole contribution of Bevan is the disclosure of biotin-16-UTP probes for HPV 16 for use in a slot blot or Southern blot (which both employ nitrocellulose filters), or in *in situ* hybridization techniques. Again, absent teachings of a DNA chip, Bevan does not supply the requisite suggestion or motivation to modify the teachings of Gravitt or combine the teachings of Gravitt or Stratagene.

Claim 31 stands rejected as obvious over Gravitt in view of Stratagene, further in view of U.S. patent **5,273,881** to Sena ("Sena"). Sena teaches the use of the fluorochrome streptavidin-R-phycoerythrin as a biotin-binding secondary detection label. Specifically, Sena teaches that the streptavidin-fluorochrome conjugate is attached to a "solid support" , *e.g.*, a *nitrocellulose* filter (col. 8, l. 16), used to capture biotin-labeled material. Sena also does not disclose use of this label on a DNA chip, nor does Sena provide the motivation to combine the references.

Claims 32-35 stand rejected as obvious over Gravitt in view of Stratagene, further in view of published U.S. patent application **2003/001295** to Shalon ("Shalon"). Shalon teaches a method of

forming DNA chips by affixing amine-linked DNA probes to aldehyde–derivatized glass surfaces, and hybridization of fluorescently labeled DNA to the probes.

According to our analysis, Shalon does not teach or suggest use of the chips for diagnosing **HPV**, nor does Shalon teach the necessity for double-labeling as taught by Gravitt and the instant invention. According to Shalon, the sensitivity of the disclosed assay is sufficient for detecting up- or downregulation of up to 10^6 genes on a single chip, and is useful to diagnose disease states based on differentially expressed gene profiles. There is no disclosure of a chip as taught by the presently claimed purpose, which purpose is not to achieve gene expression profiling, but rather, to detect the presence of HPV strains (of which there are significantly fewer than 10^6 since the claim requires about 20).

Again, there is no suggestion or motivation in Gravitt to use DNA chips *in lieu* of nitrocellulose or nylon to practice the disclosed hybridization method. As stated above, Gravitt asserts that the filter-based dot and line blotting are efficient and accurate and provide an advantage over current techniques. Since chip-based microarrays were well known in the art in 1998 when Gravitt was published, Gravitt would likely have disclosed such a modification if it was contemplated for use in conjunction with his disclosed method. Absent such a disclosure, or a disclosure in Shalon to use the chips to diagnose HPV strains in a kit and not for expression profiling (these are to very distinct goals), there can be **no motivation** to combine the references and arrive at the claimed invention.

Finally, claims 32-33, and 35 are rejected as obvious over Gravitt in view of Stratagene, further in view of **Zammatteo** et al., Anal. Biochem. 2000; 280: 143-50 ("Zammatteo"). Zammatteo discloses a method of forming microarrays using 5'amine-linked DNA probes covalently linked to an aldehyde-derivatized glass surface, and hybridization of biotinylated DNA to the array (page 148). There is no teaching in Zammatteo of arrays comprising HPV DNA probes or use for the diagnosis of HPV.

As discussed above for Shalon, there is no hint of Gravitt using aldehyde-derivatized chips *in lieu* of nitrocellulose or nylon filters, much less to modify the probes as taught in Zammatteo for covalent attachment to the filters of Gravitt. Similarly, there is no teaching in Zammatteo to use HPV specific probes, or any specific DNA, on the disclosed microarrays. Accordingly, the Examiner has not met her burden of establishing obviousness because there is no suggestion in either reference to modify the teachings to arrive at the presently claimed invention.

It is further asserted that it was well-known in 1998 to use labels for detecting DNA. However, while the present invention employs this tool of labeling, the present claims **are not** directed to a method of detecting DNA using double-labeling. The claims are specifically directed to a method for detecting HPV strains for diagnostic purposes, using an array and labeled **components**, which provides an improvement over the art since the diagnosis can be achieved using a low amount of one clinical sample. This improves the speed of the diagnosis, the accuracy of the diagnosis, and is less of a burden on the patient who must provide the sample.

In view of the above amendment, applicant believes the pending application is in condition for allowance. Accordingly allowance of all presently pending claims is respectfully requested.

Dated: October 1, 2004

Respectfully submitted,

By _____

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1: Nat Biotechnol. 1998 Jan;16(1):40-4. Related Article

DNA chips: state-of-the art.

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The technology and applications of microarrays of immobilized DNA or oligonucleotides are reviewed. DNA arrays are fabricated by high-speed robo on glass or nylon substrates, for which labeled probes are used to determine complementary binding allowing massively parallel gene expression and gen discovery studies. Oligonucleotide microarrays are fabricated either by in situ light-directed combinational synthesis or by conventional synthesis followed immobilization on glass substrates. Sample DNA is amplified by the polyme chain reaction (PCR), and a fluorescent label is inserted and hybridized to the microarray. This technology has been successfully applied to the simultaneou expression of many thousands of genes and to large-scale gene discovery, as as polymorphism screening and mapping of genomic DNA clones.

Publication Types:
- Review
- Review, Tutorial

PMID: 9447591 [PubMed - indexed for MEDLINE]

1: J Biochem Biophys Methods. 1997 Dec 3;35(3):153-9. Related Article

ELSEVIER SCIENCE FULL-TEXT ARTICLE

An improved protocol to increase sensitivity of Southern blot us dig-labelled DNA probes.

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The use of methods for nonradioactive labelling of nucleic acids has increase recent years because they avoid disadvantages associated with radioisotopes. most frequently used label is digoxigenin (DIG). The greatest problem of nonradioactive methods is their high nonspecific background mainly caused multistep detection. A diffuse background can mask the specific signal; furthermore nonspecific signals can make it difficult to interpret the result. Ir study we have attempted to identify elements which could generate backgrou We have also determined the probe and antibody concentrations by which th higher sensitivity is obtained.

PMID: 9470094 [PubMed - indexed for MEDLINE]

Display Abstract Show: 20 Sort Send to Text

High-density linkage map of rice with expressed sequence tags

B.A. Antonio, A. Shomura, T. Shimano, Y. Kuboki, S.Y. Lin, T. Inoue, H. Kajiya, Y. Harushima, K. Yamamoto, Y. Nagamura, N. Kurata, M. Yano, Y. Minobe, and T. Sasaki

We have constructed a high-density linkage map of rice using an F_2 population derived from the cross between a japonica variety, Nipponbare, and an indica variety, Kasalath. A total of 1,383 markers, which consist of cDNA clones from callus and root, genomic clones as well as RAPD markers, have been mapped covering a distance of 1,575 cM. All cDNA clones have been sequenced and searched for similarities with known proteins and can be referred to as expressed sequence tags on the map. A majority of the genomic clones and RAPD markers was also sequenced to generate sequence-tagged sites. These extensive linkage analyses gave evidence on duplication of chromosomal segments, particularly in the distal region of chromosomes 11 and 12. Additional markers are being mapped using cDNA clones derived from other cDNA libraries such as green shoot, etiolated shoot, and developing seed. Ultimately, we would like to develop a saturated linkage map that will facilitate a more efficient utilization of molecular markers for rice improvement.

One of the most important advances in the field of biotechnology, which promises to revolutionize several areas of plant genetics and breeding, is the wide utilization of molecular markers. In conjunction with phenotypic and biochemical markers, these markers will have great impact in identifying and ultimately isolating genes for various agronomically important traits. In recent years, construction of RFLP linkage maps has been reported in a number of plants (Bernatzky and Tanksley 1986, Chang et al 1988, Rognli et al 1992, Da Silva et al 1993, Kleinhofs et al 1993). In rice, a molecular linkage map covering the entire genome was developed independently by McCouch et al (1988) and Saito et al (1991) with 135 and 322 markers, respectively. Such molecular maps may provide new opportunities for application in plant genetic manipulation, particularly in tagging genes for agronomically important traits with DNA markers. In addition, these maps could also serve as important tools in understanding the evolutionary relationships among different species as shown by the

synteny studies between such crops as wheat and rye (Rognli et al 1992), potato and tomato (Tanksley et al 1992), rice and maize (Ahn and Tanksley 1993), rice and wheat (Kurata et al 1994a), etc.

In the Rice Genome Research Program (RGP), we are constructing a high-density linkage map of rice with markers spaced at very close intervals throughout the genome. Most markers in this map have been sequenced to generate expressed sequence tags and sequence-tagged sites (STSs), and as such will be a model system for overall analysis of genome structure and function in plants. So far, a map with 1,383 DNA markers at an average interval of 300 kb and distributed along 1,575 cM on the 12 linkage groups has been reported by Kurata et al (1994b). Mapping of more DNA markers is currently in progress to generate a saturated map. This paper summarizes such results as well as some of the most recent findings in restriction fragment length polymorphism (RFLP) mapping at RGP.

Materials and methods

Plant materials

The parent strains consisted of a japonica variety, Nipponbare, and an indica variety, Kasalath. A single cross was made to obtain an F_2 population and 186 individuals were used for analysis of segregation of DNA polymorphism.

DNA manipulation

Total DNA was extracted from the green leaves of parental lines as well as the F_2 progenies by the CTAB method (Murray and Thompson 1980). Then 2 μg total DNAs were each digested with one of eight restriction enzymes, *Bam*HI, *Bgl*II, *Eco*RV, *Hin*dIII, *Apa*I, *Dra*I, *Eco*RI, and *Kpn*I, overnight at 37 °C. The digested samples were applied in 0.6% agarose gel, electrophoresed for 12 h and transferred in a positively charged nylon membrane by capillary blotting. These were used for hybridization with probes labeled with horseradish peroxidase according to the protocol of ECL direct nucleic acid labeling and detection system (Amersham).

DNA probes

The probes used for hybridization consisted mainly of cDNA clones, genomic clones, and RAPD markers all derived from japonica cultivar, Nipponbare. The cDNA clones consisted of randomly selected clones from callus and root cDNA libraries. The nucleotide sequence from the 5' end for 300-400 bp was determined and translated into an amino acid sequence. Then a similarity search at the protein level was performed in the NBRF-PIR data base using the FASTA algorithm. Clones showing an optimized matching score of more than 150 with amino acid sequences in other organisms were considered as functionally identical clones. All sequenced clones are registered and deposited at the DNA Data Bank of Japan (DDBJ).

The genomic clones used for mapping consisted of random genomic clones, YAC-end clones, *Not*I linking clones, and telomere-associated sequences (TELs). The random genomic clones were prepared by ligating *Hin*dIII or *Pst*I DNA fragments in

pBluescriptII SK+ or pUC vector. The YAC-end clones were derived from both ends of a large size DNA fragment cloned in YAC, amplified by PCR as 200-1000 bp long DNA, and ligated into TA cloning vector PCR™1000. The *Not*I linking clones consisted of *Sau*3AI partially digested 500-4000 bp fragments with *Not*I sites and cloned in pT7T318U vector at the *Bam*HI site. The TELs were obtained using cassette ligation-mediated PCR of *Sau*3A1 DNA digests and cloned in pCRII vector (Ashikawa et al 1994). For mapping of RAPD markers, 60 arbitrarily designed 10-nucleotide primers were initially subjected to RAPD analysis. Then, these primers were paired randomly and were used for detection of RAPD markers. Detection and mapping of RAPD markers and conversion of RAPD to STS markers were described by Monna et al (1994).

Linkage analysis

The segregation patterns and linkage relationships of RFLP in the F_2 population were analyzed using the MAPMAKER/EXP 3.0 software (Lander et al 1987). Multipoint analysis was performed to calculate the linkage of a large number of markers and produce a map of their order along the chromosomes. Recombination values between the markers were transformed into centimorgan (cM) distance by the Kosambi function (Kosambi 1944).

Results and discussion

RFLP map with 883 expressed sequences

To construct an RFLP linkage map of rice, we analyzed 2,950 cDNA clones from callus and root cDNA library. These clones showed various banding patterns such as single bands, double bands, as well as multiple bands with a smeared background in some cases, suggesting either single-copy sequences or repeated sequences in the genome. A total of 883 cDNA clones, which consisted of 465 clones from callus cDNA and 418 clones from root cDNA, showed distinct RFLP and were used for segregation analyses of the F_2 population derived from the cross Nipponbare/Kasalath. The positions of these clones represented by C-number and R-number for callus and root cDNA clones, respectively, are shown in Figure 1. A more detailed version of this map appeared in Kurata et al (1994b) and included such information as the accession number of the sequence data deposited in the DDBJ. In addition to cDNA clones, 265 genomic DNAs, 147 RAPD markers, and 88 other DNAs were also mapped for a total of 1,383 markers distributed along 1,575 cM on 12 linkage groups at an average interval of 1.14 cM.

A similarity search for proteins of other organisms showed that the cDNA clones have a high similarity to genes of a wide range of organisms including dicots, monocots, mammals, and yeast (Table 1). Most of these genes code for isozymes such as alcohol dehydrogenase (*adh*), aspartate aminotransferase (*got*), fructose bisphosphate aldolase (*ald*), glucose-6-phosphate isomerase (*pgi*), peroxidase (*pox*), etc. In the conventional linkage map, several isozymes have been mapped and assigned to specific chromosomes (Wu et al 1988). In our RFLP linkage map, we determined the loci of

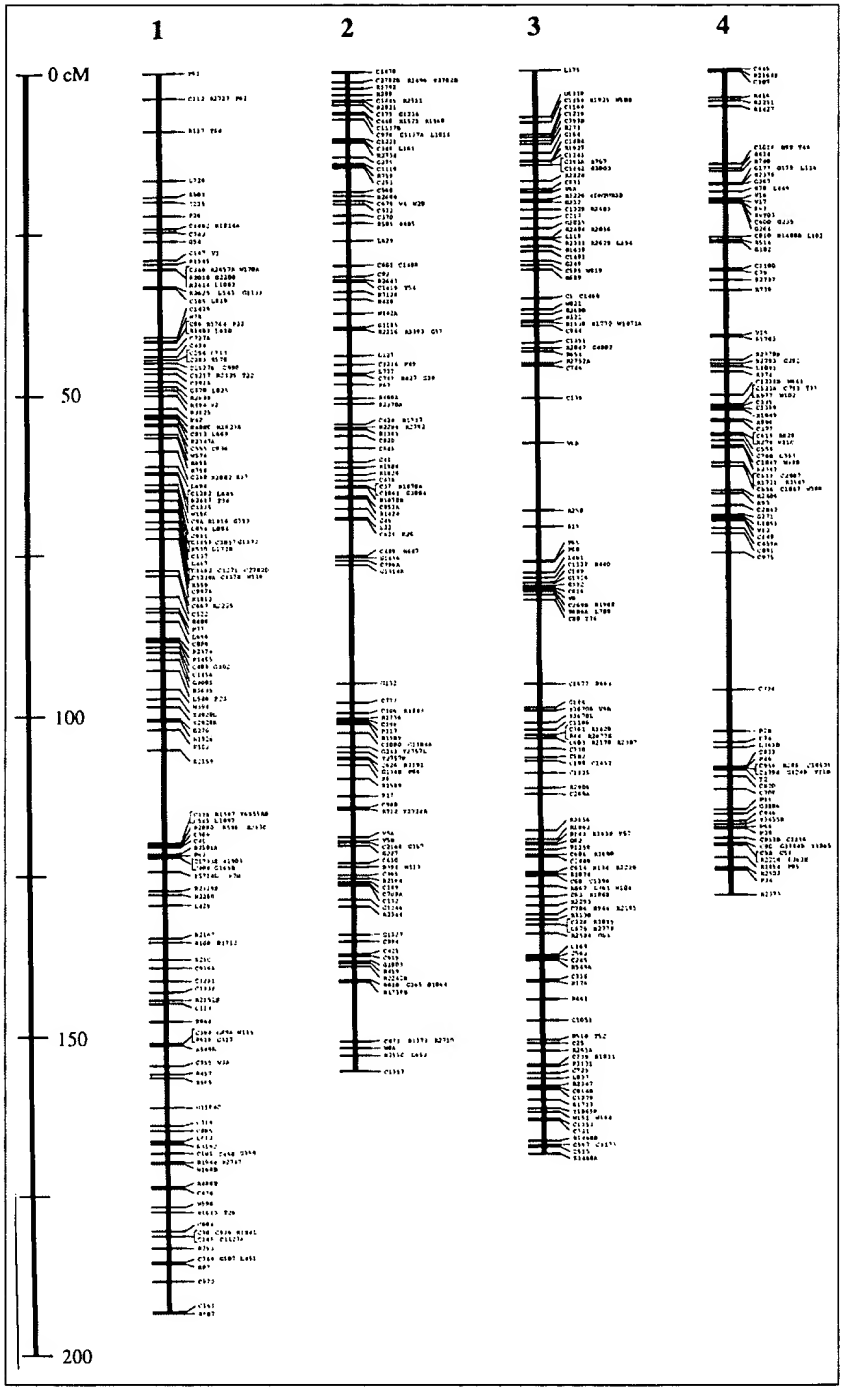
1
2
3
4
0 cM
50
100
150
200

Fig. 1. An RFLP linkage map of rice constructed with 1,383 DNA markers. The markers are designated as follows: C, clones from callus cDNA library; R, clones from root cDNA library; G, random genomic clones; L, *Not* I linking clones; Y, YAC-end clones (L and R after the Y-number indicate left and right end clones); P, RAPD markers; T, RAPD markers converted to STS; TEL, telomere-associated sequences; W, wheat clones; and V, clones from sources other than RGP.

Figure 1 continued

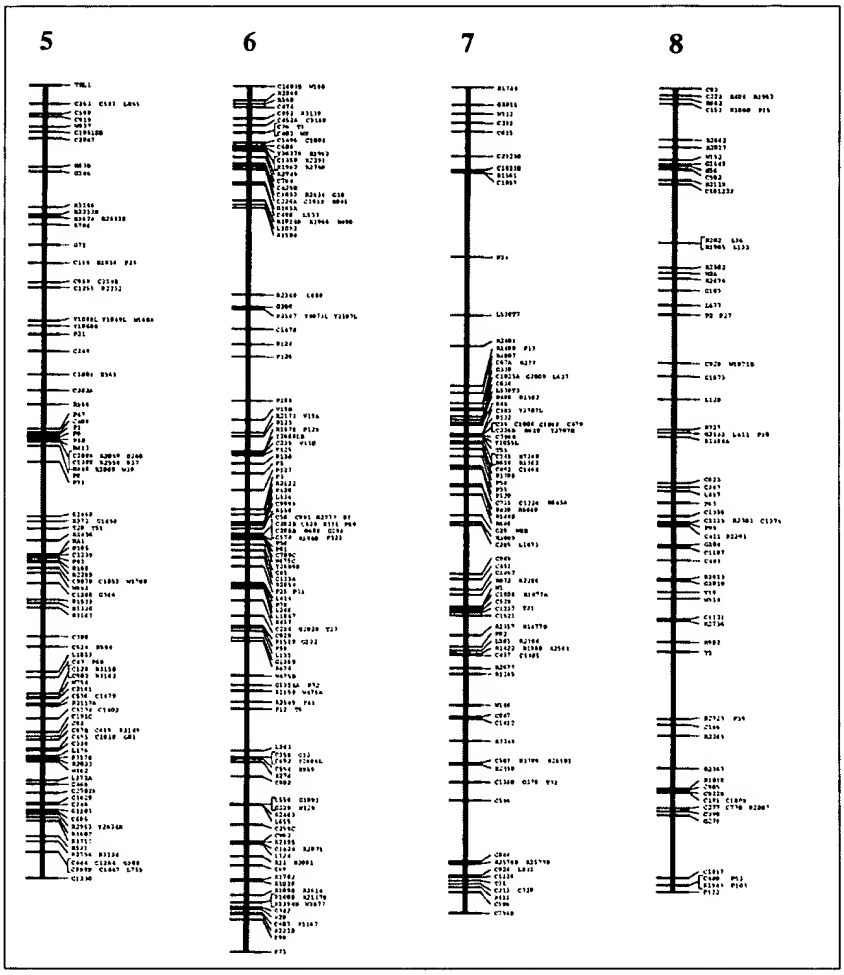

Figure 1 continued

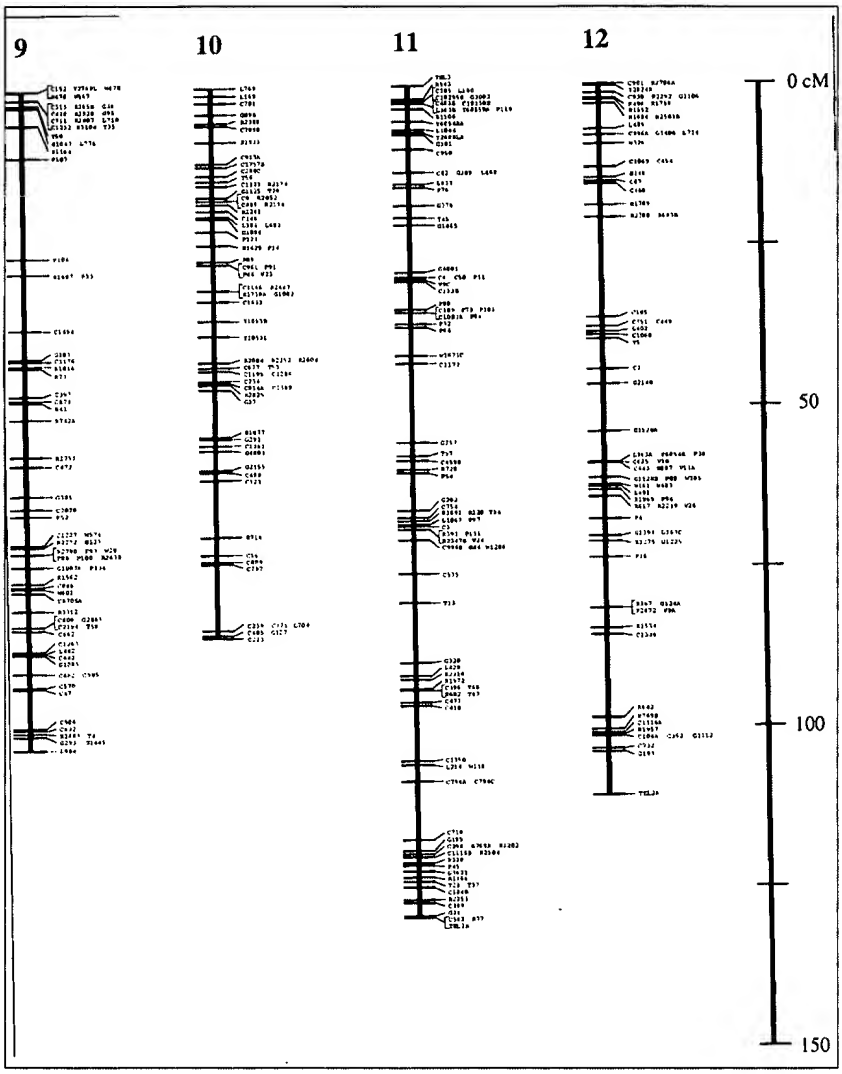

Table 1. Callus and root cDNA clones mapped in rice with similarity to known proteins.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
1	30.4	R2657A	*ald2*	Fructose bisphosphate aldolase	Rice	D28322
1	41.8	R1764	*got2*	Aspartate aminotransferase	*Bacillus sp.*	D24345
1	42.9	C727A	*gco1*	Glucan endo-1,3-beta-glucosidase	Common tobacco	D15500
1	44.5	C256	*rcs*	Reductase	Soybean	D15203
1	44.5	R578	*hbo*	(S)-tetrahydroberberine oxidase	*Coptis japonica*	D23922
1	49.3	R494	*nia*	Nitrate reductase (NADH)	Tomato	D23879
1	53.1	R1623S	*tub3*	Tubulin beta-2 chain	*Arabidopsis thaliana*	D24277
1	58.9	C250	*got1*	Aspartate aminotransferase	Proso millet	D23735
1	58.9	R37	*glt1*	Glutathione transferase 1	Maize	D32736
1	63.6	C9A	*elf3*	Elongation factor 2	*Caenorhabditis elegance*	D15078
1	64.5	C911	*gtl*	Glutamin:tRNA ligase	Human	D15594
1	69.2	R559	*ppp*	Phosphoprotein phosphatase	Human	D23910
1	70.3	C922A	*gbp*	GTP-binding regulatory protein beta chain	*Chlamydomonas reinhardtii*	D22667
1	71.9	R1012	*lcl*	Long-chain-acid:CoA ligase	Human	D24049
1	81	R886	*mdh*	Malate dehydrogenase, mitochondrial	Water melon	D24025
1	84.7	C808	*eif2*	Initiation factor eIF-4A	Curled-leaved tobacco	D22665
1	87.7	C409	*sip*	Stress inducible protein STI1	Yeast	D15287
1	90.4	R2635	*soi*	Spil hypothetical protein	Yeast	D24836
1	99.9	R1928	*vcp*	Vasoline-containing protein	Pig	D28306
1	119.1	C585	*secl*	SEC 7 protein	Yeast	D15403
1	119.4	R2630	*hud*	Elav/Sex-lethal related protein	Human	D24832
1	119.4	R596	*glt2*	Glutathione transferase 1	Maize	D28287
1	119.4	R2880	*osb*	Oxysterol-binding protein	Rabbit	D24980
1	119.5	C369	*gdh*	Glutamate dehydrogenase (NAD(P)+)	*Halobacterium salinarium*	D15259
1	121.3	C904	*sall*	SalT protein precursor	Rice	D28208
1	126.4	R476	*ams1*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D28266
1	126.9	R2280	*ams4*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D24629
1	133.7	R2167	*ams3*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D28314
1	137	R210	*cad1*	Cathepsin D	Human	D23806
1	142.1	C1338	*ang*	58K antigen	*Ricketssia tsutsugamushi*	D22792
1	149.6	C399	*idh*	Isocitrate dehydrogenase (NADP+)	Alfalfa	D15280
1	155.3	R665	*rac1*	Rac1 protein	Human	D23963
1	165.7	R3192	*spk*	Serine/threonine-specific protein kinase	*Arabidopsis thaliana*	D25110
1	172.4	R480B	*ypt*	Transforming protein, ypt 1, homolog	Maize	D23874
1	180.3	C936	*mtn*	Metallothionein-like protein	*Arabidopsis thaliana*	D15602
1	180.3	C30	*tpi*	Triose phosphate isomerase	Maize	D15092
1	181.6	R753	*sds*	C-5 sterol desaturase	Yeast	D23996
1	184.1	R87	*tin*	Trypsin inhibitor	Rice	D23762

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
2	1.6	R2702B	*hsp5*	Heat shock protein 70	Common tobacco	D23418
2	4.4	C1445	*aux*	Auxin-induced protein	*Arabidopsis thaliana*	D15870
2	6.3	C440	*dfr1*	Dihydrofolate-4-reductase	Garden petunia	D15312
2	6.6	C1137B	*dfr2*	Dihydrofolate-4-reductase	Garden snapdragon	D15715
2	7.4	C1137A	*dfr2*	Dihydrofolate-4-reductase	Garden snapdragon	D15715
2	32.3	C92	*ant*	Adenine nucleotide translocator	Rice	D22519
2	33.1	C1419	*thr*	Thioredoxin reductase (NADPH)	*Escherichia coli*	D13855
2	34.6	R3128	*eno2*	Enolase	Tomato	D25085
2	40.5	R3393	*clc*	Clathrincoat assembly protein	Rat	D24586
2	51.2	R480A	*ypt*	Transforming protein, ypt1, homolog	Maize	D23874
2	55.3	R1737	*prs*	Proteasome XC3 chain	African clawed frog	D24326
2	55.8	R2284	*ams5*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D24632
2	63.3	R1826	*nab*	X16 protein	Mouse	D24389
2	65	C37	*gpdl*	Glyceraldehyde-3-phosphate dehydrogenase	White mustard	D15096
2	67	R1424	*ste1*	Regulatory protein STE7	Yeast	D24144
2	70.3	C621	*reg1*	14-3-3 protein	Barley	D15430
2	75.9	R447	*sac*	SAC1 protein	Yeast	D23860
2	103.4	C1000	*hsp3*	Heat shock protein 70	Maize	D15636
2	107.2	C626	*cyc*	cyc07 protein, S-phase specific periwinkle	Madagascar	D15433
2	120.6	C2168	*got3*	Aspartate aminotransferase	Proso millet	D16037
2	138.2	C915	*stk*	Kinase-related transforming protein	Mouse	D15597
2	139.3	R459	*gdc2*	Glycine-cleavage system protein H	Garden pea	D23865
2	139.8	R2242S	*tub4*	Tubulin beta-2 chain	Garden pea	D24606
2	142	R810	*ubq4*	Ubiquitin	Garden snapdragon	D25349 D25350
2	151.4	R2710	*urt2*	UTP:glucose-1-phosphate uridyltransferase	Potato	D24887
3	14.7	R707	*qpc*	Ubiquinone binding protein QP-C	Bovine	D23977
3	18.5	C831	*rad6*	RAD6 DNA-repair homolog *Dhr6*	Fruit fly	D22670
3	20.1	R3226	*cof*	Cofilin	Yeast	D25113
3	21.7	R2443	*myb*	Transforming protein, myb, homolog	Maize	D24724
3	21.7	C1329	*pgi*	Glucose-6-phosphate isomerase	*Clarkia lewesii*	D15815
3	26.1	R2856	*cak*	Casein kinase II alpha chain	Maize	D24965
3	26.1	R2404	*eif4*	Initiation factor eIF-5A	Common tobacco	D24702
3	26.3	R2628	*tpa*	Transplantation antigen P198	Mouse	D24830
3	35.4	C1468	*tub2*	Tubulin alpha-2 chain	Maize	D15886
3	37.9	R2690	*act*	Actin 1	Rice	D24576
3	39.2	R1538	*reg4*	14-3-3 protein	Barley	D24218
3	43.2	R2847	*gco2*	Beta-glucosidase	White clover	D24959
3	45.9	C746	*gri*	Glycine rich protein 2	*Arabidopsis thaliana*	D15512
3	79.7	C549	*hsp1*	Heat shock protein 70	Spinach	D22613

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
3	81.2	R1908	*acb*	Endozepine	Yeast	D28303
3	103.7	R2170	*uqn*	NADH dehydrogenase (ubiquinone) chain 2	*Paramecium tetraurelia*	D28315
3	107.2	C1452	*sod*	Superoxide dismutase	Rice	D15675
3	119.6	R1862	*prp*	Prp 16-1 protein	Yeast	D24417
3	121.2	R1158	*snr*	Small nuclear RNA-associated protein	Human	D24080
3	122	R1690	*eif3*	Initiation factor 2 alpha chain	Yeast	D24301
3	128.5	C63	*ubq1*	Ubiquitin fusion protein	Fruit fly	D15108
3	134.2	R2584	*cdh*	Cinnamyl-alcohol dehydrogenase	Kidney bean	D14802
3	150.7	R518	*elf1*	Elongation factor 1 alpha	Tomato	
3	160	R1713	*glt3*	Glutathione transferase III	Maize	D24311
3	166.5	R1468A	*cdc*	CDC2a protein	Rice	D24174
4	4.7	R416	*aox*	Amine oxidase	Rat	D23854
4	15.5	R634	*ocp*	Oryzain alpha chain	Rice	D23944
4	16	R740	*gyk*	Glycerol kinase	*Bacillus subtilis*	D23993
4	19.3	R78	*kin*	ncdD protein	Fruitfly	D23757
4	53.3	R1849	*art*	Arabinose transport protein	*Escherichia coli*	D24407
4	54.6	R896	*gpd2*	Glyceraldehyde-3-phosphate dehydrogenase	Maize	D28294
4	57.4	C559	*ppa*	Inorganic pyrophosphatase	Yeast	D15382
4	59	C1047	*reg3*	14-3-3 protein	Barley	D15663
4	109.2	R288	*ccp*	Cytochrome C peroxidase	Yeast	D23832
4	109.2	C954	*dds*	Dihydrodipicolinatesynthase	Wheat	D15614
4	109.2	C1794	*his1*	Histone H1	Wheat	D22924
4	121.3	C9B	*elf3*	Elongation factor 2	*Caenorhabditis elegance*	D15078
5	27.9	R1838	*dnj*	dnaJ protein homolog	Human	D24399
5	30.9	C259B	*ubq2*	Ubiquitin	Tomato, potato, oat	D22550
5	45	R569	*omc*	2-oxoglutarate/malate carrier protein	Bovine	D23915
5	55.5	R2059	*rbp*	Ribophorin	Human	D24495
5	55.5	C1388	*rab11*	GTP-binding protein rab11	Dog	D15842
5	55.5	R2558	*acc*	Acetyl-CoA carboxylase	Yeast	D24786
5	95.2	R3182	*hsp6*	Heat shock protein cognate 70	Tomato	D25105
5	95.2	C128	*ubc*	Ubiquitin conjugating protein	Wheat	D15130
5	96.8	C536	*pdc*	Pyruvate decarboxylase	Maize	D15369
5	102.2	C67B	*rif*	ADP-ribosylation factor 4	Human	D22513
5	102.2	C419	*cam*	Calmodulin	Wheat	D15295
5	109	C466	*mpp*	Processing peptidase catalytic chain, mitochondrial	Yeast	D15329
5	113.4	C686	*atp1*	H+-transporting ATP synthase beta chain	Rice	D15470
5	113.7	R2953	*dyl*	Dynamin-like protein	Fruit fly	D25026
5	118	R2754	*cad2*	Cathepsin D	Human	D24912
5	119.6	C1264	*kri*	Ketol-acid reductoisomerase chloroplast	Spinach	D27768
6	2.2	R2869	*pgd*	Phosphogluconate dehydrogenase	*Synechococcus* sp.	D24970
6	9.2	C688	*prt*	Transcription factor for E3	Human	D15472
6	9.8	R2291	*ste2*	Regulatory protein STE7	Yeast	D24636
6	10.1	R2749	*cys*	Cysteine synthase B	Pepper	D24907

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
6	11.2	C764	*hca*	ClassII histocompatibility antigen	Human	D15525
6	12.6	C1032	*ag12*	Floral homeotic protein AGL2	*Arabidopsis thaliana*	D15657
6	13.1	R845	*ctl*	Cystathionine gamma-lyase	Yeast	D28293
6	17.9	R1966	*sus*	Sucrose synthase	Barley	D24462
6	34.8	R2147	*sal2*	SalT protein	Rice	D24547
6	57	C235	*hmg2*	High mobility group-like protein NHP2	Yeast	D15191
6	69.8	R111	*fdh*	Formate dehydrogenase	*Pseudomonas* sp.	D23770
6	69.8	C58	*srp*	Signal recognition particle 19K	Human	D15105
6	112	C556	*gdc1*	Glycine-cleavage system protein H	Garden pea	D15379
6	112.1	R2403	*pgk*	Phosphoglycerate kinase, cytosolic	Wheat	D26320
6	115.2	C259C	*ubq2*	Ubiquitin	Tomato, potato, oat	D22550
6	121.5	C69	*eifl*	Initiation factor eIF-4A	Curled-leaved tobacco	D15109
6	126.2	R1888	*ams2*	S-adenosylmethionine synthetase 2	*Arabidopsis thaliana*	D24436
6	127.3	R1394B	*nod*	Nodulation protein	*Rhizobium legumino-sarum*	D24124
6	128.9	R1167	*cat*	Catalase chain I	Maize	D24082
6	128.9	C607	*hmg1*	High mobility group protein	Wheat	D28196
7	40.3	R2401	*thx*	Thioredoxin	*Arabidopsis thaliana*	D24700
7	46.5	R1488	*hxk*	Hexokinase P1	Yeast	D24182
7	49.2	C67A	*rif*	ADP-ribosylation factor 4	Human	D28199
7	54.2	R610	*mak*	MAK16 protein	Yeast	D23935
7	54.2	C479	*sps*	Spermidine synthetase	Human	D22594
7	55.4	C492	*gcw3*	Glysine-rich cell wall structural protein	Garden petunia	D22596
7	88	R2394	*cpk*	Protein kinase, calcium dependent	Soybean	D24697
7	98.5	C1412	*elf2*	Elongation factor1 beta chain	Rice	D15852
7	101.9	R3349	*cyt*	Cystathionine gamma-lyase	Potato	D25146
7	105.3	C507	*cpn*	Probable chaperonin	*Synecho-coccus* sp.	D26192
7	108.4	C1340	*par*	Par gene protein	Common tobacco	D22794
7	124.1	C213	*odh*	Oxoglutarate dehydrogenase	*Escherichia coli*	D15178
7	124.6	R411	*tab*	Tat-binding protein	Human	D23852
7	125.4	C586	*gcw1*	Glycine-rich cell wall structural protein	Garden petunia	D22623
8	1.1	R1963	*map*	Membrane alanyl aminopeptidase	*Escherichia coli*	D28310
8	1.8	R662	*hyp2*	Hypothetical protein 1 (sul 3' region)	*Bacillus subtilis*	D23961
8	2.6	R1880	*acl*	Acyl carrier protein 3	Barley	
8	23.5	R1985	*pkc2*	Protein kinase C homolog	Rice	D24464
8	27.9	R2382	*pat*	Patatin T5	Potato	D24690
8	42.5	C929	*reg2*	14-3-3 protein	Barley	D22692
8	53.9	R1394A	*nod*	Nodulation protein	*Rhizobium legumino-sarum*	D24124

Table 1 continued.

Chr.	Position	Marker	Gene	Protein name	Organism	DDBJ ID No.
8	100.5	R2285	*gdh*	Glucose dehydrogenase (pyrroloquinoline-quinone)	*Acinetobacter calcoaceticus*	D24633
8	109.1	C922B	*gbp*	GTP-binding regulatory protein beta chain	*Chlamydomonas reinhardtii*	D22667
8	111.7	C277	*rpa*	Acidic ribosomal protein 4	Fruit fly	D15212
9	0.8	C711	*pab*	Polyadenylate-binding protein	Human	D15488
9	46.7	C397	*sco1*	SCO1 protein	Yeast	D22575
9	74.6	R1562	*hsp4*	Heat shock protein 82	Rice	D24234
9	75.1	C846	*pkc1*	Protein kinase C homolog	Rice	D15569
9	78.7	R3312	*gco3*	Beta-glucosidase B	*Bacillus polymyxa*	D28326
9	88.4	C985	*hsp2*	Heat shock protein 82	Rice	D22707
9	97	C506	*hmg3*	High mobility group protein	Maize	D22603
9	97.3	C632	*urt1*	UTP:glucose-1-phosphate uridyltransferase	Potato	D15437
10	2.3	C701	*adh2*	Alcohol dehydrogenase	Human	D15481
10	11.7	C913A	*eno1*	Enolase	Tomato	D28210
10	17.6	C489	*atp2*	H+-transporting ATP synthase gamma chain	*Rhodospirilum rubrum*	D15343
10	42.7	R2604	*gcw4*	Glycine-rich cell wall structural protein	Rice	D24186
10	42.7	R2252	*hyp4*	Hypothetical protein YCL59C	Yeast	D24612
10	43.5	C677	*gcw2*	Glycine-rich cell wall structural protein	Rice	D13464
11	9.2	C950	*tum*	Tumor protein	*Arabidopsis thaliana*	D22697
11	65	R120	*ahc*	Adenosyl homocysteinase	Rat	D23773
11	65.8	C3	*sec2*	Sec23 protein	Yeast	D22492
11	91	R1572	*adh2*	Alcohol dehydrogenase	Rice	D24243
11	91.3	C496	*adh1*	Alcohol dehydrogenase	Maize	D15347
11	91.3	R682	*adh2*	Alcohol dehydrogenase	Maize	D23967
11	114	R3202	*cbp*	Calcium binding protein	Mouse	D25111
12	1.4	R2292	*rab5*	GTP-binding protein rab5	Dog	D28317
12	14.5	C1069	*hyp1*	Hypothetical protein	Maize	D15675
12	72.6	R3375	*cla*	Clathrin-associated protein 17	Rat	D25151
12	83	R2672	*elf4*	Elongation factor selB	*Escherichia coli*	D24864
12	87.1	C1336	*ald1*	Fructose-biphosphate aldolase	Rice	D28223
		15 mapped	*pox*	Peroxidase	Horseradish and turnip	
		3 mapped	*his2a*	Histone H2A	Mainly wheat and maize	
		4 mapped	*his2b*	Histone H2B	Mainly wheat and maize	
		4 mapped	*his3*	Histone H3	Mainly wheat and maize	
		5 mapped	*his4*	Histone H4	Mainly wheat and maize	
		24 mapped	*rpl*	Ribosomal protein large subunit	Mainly rat	
		15 mapped	*rps*	Ribosomal protein small subunit	Mainly rat	

these isozymes by mapping cDNA clones derived from callus and root cDNA libraries. Thus, such genes as *got*, *adh*, and *pox*, which have been assigned in the conventional linkage map by segregation analysis of gene products, could be accurately mapped with their exact locations in the chromosome. In addition, a number of genes, which code for structural proteins such as actin, tubulin and ubiquitin, genes associated with the glycolytic pathway, genes related to the cell cycle, as well as heat shock proteins,

were also mapped. Some of these genes, however, did not necessarily correspond to a specific gene sequence but rather to one of the highly conserved multiple copies in the genome and were mapped in several loci in one or more chromosomes.

Several multigene families such as ribosomal proteins and histones, which have been identified from the large-scale cDNA analysis, have also been mapped. Twenty-four genes of the large subunit ribosomal protein and 15 genes of the small subunit ribosomal protein were found to be widely distributed in the rice genome. We have also identified and mapped the genes for histone proteins, namely, H1, H2A, H2B, H3, and H4 proteins. In human and other animals, these five types of genes formed clusters or repeated tandem units. In rice, however, they were found to be widely distributed in several chromosomes.

Thus, construction of a detailed genetic map using expressed gene sequences may provide a vast amount of information on the structural and functional organization of the rice genome. This could be very useful in identifying a gene of interest as well as in the subsequent stage of manipulation and isolation.

Genomic DNA markers as sequence-tagged sites

The chromosomal distribution of genomic clones classified as random genomic clones (G-number), *Not*I linking clones (L-number), YAC-end clones (Y-number), and TELs were also determined (Fig. 1). One hundred and thirty-seven randomly selected genomic clones were evenly distributed on the map. Most of these genomic clones have been sequenced and registered at DDBJ. Thus, these clones can be referred to as STSs on the map. The YAC-end clones and *Not*I linking clones were used for mapping to determine the nature of these sequences, which was necessary for physical map construction. However, mapping of 33 YAC-end clones (Y-number) and 90 *Not*I linking clones (L-number) did not show any specific features in terms of distribution and chromosomal localization of these clones. Among the mapped YAC-end clones were those containing both ends of the DNA fragment in YAC. These clones were mapped at close proximity to each other so that the physical distance corresponding to the genetic distance in cM can be calculated.

The map positions of TELs isolated using cassette ligation-mediated PCR were also determined (Ashikawa et al 1994). Two of these clones have been located on opposite ends of chromosome 11 so that this chromosome could be completely saturated with DNA markers. Subtelomeric clones have also been mapped on one end of chromosome 12 as well as chromosome 5.

RAPD markers were used to fill such regions on the map with very few markers. More than 150 RAPD were detected between Nipponbare and Kasalath using 1,400 combinations of arbitrarily designed 10-nucleotide primers (Monna et al 1994). One hundred and forty-seven RAPD markers represented by P-number and T-number on the map were mapped on the 12 chromosomes of rice. The T-number markers correspond to RAPD markers, which were converted to STS. More importantly, regions in some chromosomes that cannot be linked by DNA markers had been successfully connected by RAPD markers. The distal regions of chromosomes 1, 6, and 8 were extended by RAPD markers P61, P73, and P122, respectively. These suggest that

RAPD markers can be very useful to fill gaps or to extend the linkage map of each chromosome.

Synteny with the wheat genome

To clarify the relationships of the rice genome with other crops, 60 wheat genomic DNA fragments (W-number) have been mapped on our high-density linkage map in collaboration with the Cambridge Laboratory, John Innes Centre, UK. The results showed that most of these markers have the same linkage order in wheat and rice (Kurata et al 1994a). Furthermore, it has been clarified that rice chromosome 1 corresponds to wheat group 3, rice chromosome 2 to wheat 6, rice 3 to wheat 4, rice 4 and 7 to wheat 2, rice 5 to wheat 1, rice 6 to wheat 7, and rice 9 to wheat 5. This suggests conservation of genome structure between rice and wheat, which are from different Gramineae tribes and differ in both chromosome number and genome size. We are also pursuing reciprocal mapping of DNA probes with other crops such as barley and maize. Eventually, we hope to clarify the extent of synteny and linkage conservation among cereal crops.

Conserved linkage order in chromosomes 11 and 12

Although most of the clones used as probes showed a single-copy band on genomic Southern hybridization, some DNA probes had two or more bands and were located in duplicate or triplicate loci. Seventy-nine probes (6.1% of the total mapped DNA probes) were mapped on more than one locus. Duplicate segments were particularly observed between chromosomes 11 and 12 (Nagamura et al 1995, Fig. 2). Thirteen of the 33 mapped DNA markers at the distal regions of these chromosomes, including a TEL (TEL2), were mapped as duplicate loci. These duplicated segments occupy 10 and 11.8 cM in chromosomes 11 and 12, respectively. The other 20 markers in these regions also showed two or more main bands, but only one band was polymorphic, which was mapped in either chromosome 11 or 12. This suggests that RFLP mapping can also be an effective method to clarify chromosomal rearrangements as well as conservation of gene order accompanied by the evolution of a species.

Toward a saturated linkage map and more

At present, we are mapping additional markers in our RFLP linkage map to create a tighter linkage. In addition to callus and root, we are also using cDNA clones from green shoot, etiolated shoot, and developing seed cDNA libraries. As of Mar 1995, we have mapped an additional 521 DNA markers so that our map now has 1,904 DNA markers and a length of 1,556 cM. The average interval between markers is about 0.8 cM. However, there are still several regions in some chromosomes with very few markers as well as long stretches without any markers. Thus, it is necessary to screen for more markers to fill these gaps or to analyze the exact nature of such regions in the chromosomes.

Ultimately, we would like to establish a map with about 2,000 DNA markers at very close intervals necessary for physical map construction and gene tagging. Selection and ordering of YAC clones covering the entire genome to construct a detailed physical

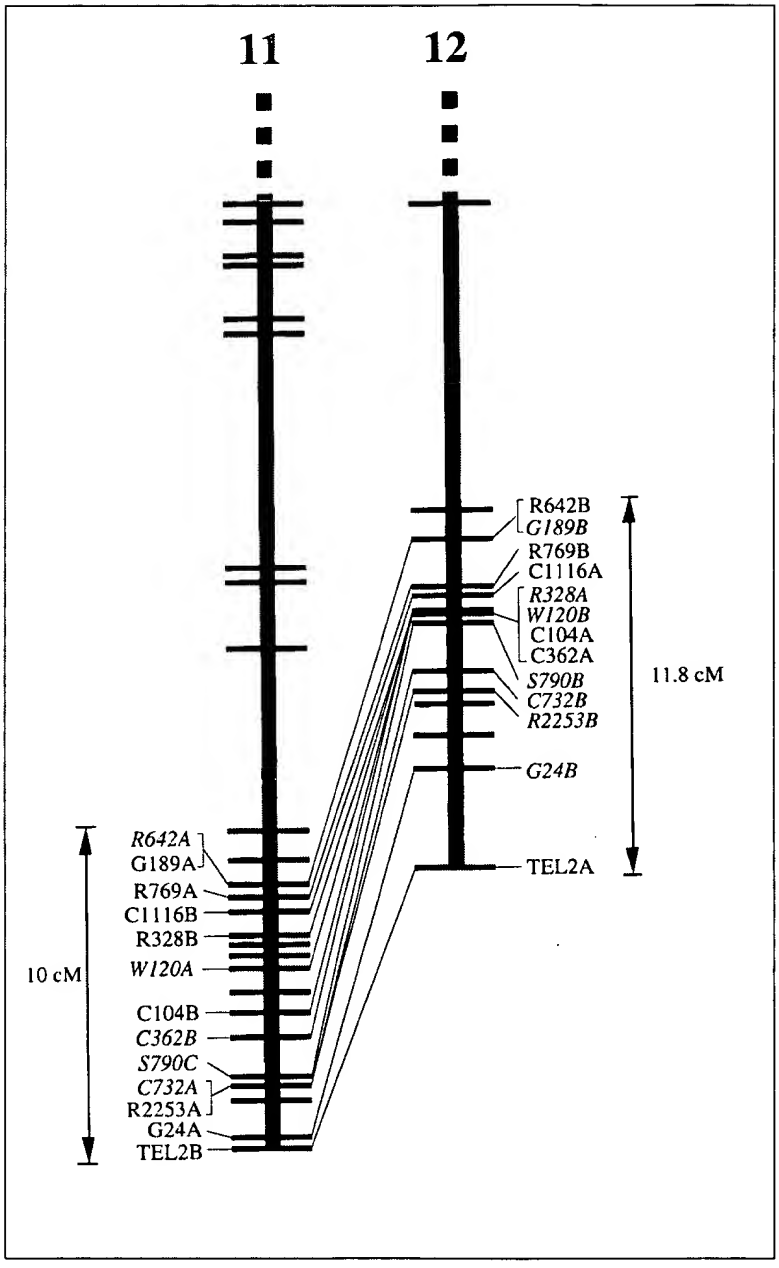

Fig. 2. The distal region of chromosomes 11 and 12 with highly conserved linkage of 13 DNA markers. Marker designations are described in Figure 1. Markers in italics were mapped after the publication of the linkage map in Kurata et al (1994b).

map of rice is in progress. Tagging of genes controlling phenotypical traits, which are important agronomically and for scientific studies, is also under way. We have already identified the chromosomal locations of such genes as *Xa1* (bacterial blight resistance gene) and *Se1* (photoperiod sensitivity gene). Isolation of these genes is expected to progress efficiently through positional map-based cloning with tagged DNA markers by using physically arrayed YAC or cosmid clones.

Thus, a high-density linkage map of rice will have far-reaching applications in understanding genome organization, function, and evolution. More importantly, it is expected to have enormous impact on the more practical aspect of plant genetic manipulation, that is, for marker-aided selection in breeding programs as well as for map-based cloning of agronomically important genes.

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Notes

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Citation: [IRRI] International Rice Research Institute. 1996. Rice genetics III. Proceedings of the Third International Rice Genetics Symposium, 16-20 Oct 1995. Manila (Philippines): IRRI.